CONFOCAL LASER SCANNING MICROSCOPY: A NEW TOOL FOR STUDYING THE EFFECTS OF CIGUATOXIN (CTX-1B) AND D-MANNITOL AT MOTOR NERVE TERMINALS OF THE NEUROMUSCULAR JUNCTION IN SITU

JORDI MOLGO, PASCAL JUZANS AND ANNE MARIE LEGRAND

Molgó, J., Juzans, P. & Legrand, A.M. 1994 08 01: Confocal laser scanning microscopy: a new tool for studying the effects of ciguatoxin (CTX-1B) and D-mannitol at motor nerve terminals of the neuromuscular junction in situ. *Memoirs of the Queensland Museum* **34**(3): 577–585. Brisbane. ISSN 0079-8835.

The confocal laser scanning microscope was used in conjunction with the fluorescent probe FM1-43 to study the effects of ciguatoxin (CTX-1B) and D-mannitol at motor nerve terminals of the neuromuscular junction in situ. CTX-1B caused time-dependent changes in the surface area of motor nerve terminals and perisynaptic Schwann cell at living neuromuscular junctions.These changes were completely prevented by tetrodotoxin indicating that they are related to both entry of Na^+ and increased quantal acetylcholine release. D-mannitol at concentrations reported to exert an effective hydroxyl radical scavenger action neither prevented the action of CTX-1B nor antagonized its effects. However, at higher concentrations D-mannitol exerted osmotic effects that caused shrinkage of both motor nerve terminals and Schwann cell somata previously swollen by the action of CTX-1B probably by shifting water from the intracellular to the extracellular compartment.

Jordi Molgó and Pascal Juzans, Laboratoire de Neurobiologie Cellulaire et Moléculaire, Centre National de la Recherche Scientifique, 91198-Gif sur Yvette Cedex, France; Anne Marie Legrand, Institut Territorial de Recherches Médicales Louis Malardé, Associé à l'Institut Pasteur, B. P. 30 Papeete, Tahiti, Polynésie Française; 2 February,1994.

Ciguatoxins (CTX) are a family of potent lipid-soluble cyclic polyethers (Scheuer et al., 1967; Tachibana et al.,1987; Legrand et al., 1989; Murata et al.,1989,1990; Lewis et al., 1992; Lewis & Sellin,1991) involved in ciguatera fish poisoning (Anderson & Lobel, 1987; Russell & Egen,1991; Swift & Swift, 1993). The poisoning is characterized by severe gastrointestinal and neurological disturbances (Bagnis et al.,1979; Withers,1982; Gillespie et al.,1986) which develop after consumption of coral reef fish.

The chemical structures (Murata et al., 1989, 1990; Lewis et al.,1991) of structurally related ciguatoxins (CTX-1B or CTX-1 or CTX, which is probably the major toxin involved in ciguatera, CTX-2 and CTX-3) are reminiscent of brevetoxins, (Baden,1989; Murata et al., 1989, 1990; Lewis et al.,1991; Gawley et al., 1992) and they share a common binding site with the brevetoxins on the neuronal voltage-sensitive sodium channel proteins (Lombet et al.,1987; Lewis et al.,1991).

CTX selectively acts on Na^+ channels in the node of Ranvier of single myelinated nerve fibers in such a way that voltage-clamped Na^+ channel currents are activated at potentials about 30mV more negative than unmodified channels and fail to inactivate during long-lasting depolarizations (Benoit et al.,1986). It is likely that a persistent activation of Na^+ channels by CTX at the resting membrane potential leads to a membrane depolarization and the spontaneous action potentials reported on neuronal, axonal and muscle membranes (Benoit et al.,1986; Bidard et al., 1984; Molgó et al.,1990). Tetrodotoxin (TTX) which blocks voltage-gated sodium channels in those membranes, prevents such actions (Benoit et al.,1986; Bidard et al., 1984; Molgó et al., 1990). CTX has also been reported to increase intracellular Ca^{2+} concentration in NG108-15 hybrid cells bathed in standard medium or in a Ca^{2+}-free medium supplemented with EGTA (Molgó et al.,1992a,1993b). CTX-induced Ca^{2+} mobilization prevents further effect of bradykinin (1μM) suggesting that CTX also stimulates the inositol 1,4,5-trisphosphate-releasable Ca^{2+} store (Molgó et al.,1993b). Since TTX prevents the CTX-induced increase in intracellular Ca^{2+} concentration it would appear that Na^+ influx through voltage-gated Na^+ channels somehow leads to release of intracellular Ca^{2+}. Such a direct relationship of Na^+- dependent Ca^{2+} mobilization in neuronal cells is unknown.

CTX increases the rate of release of [^{3}H]-aminobutyric acid and [^{3}H] dopamine from rat brain synaptosomes. These actions are sensitive to blockade by TTX but are unaffected by Ca^{2+} channel antagonists like nitrendipine and D-600 (Bidard et al.,1984). Since CTX has no action on

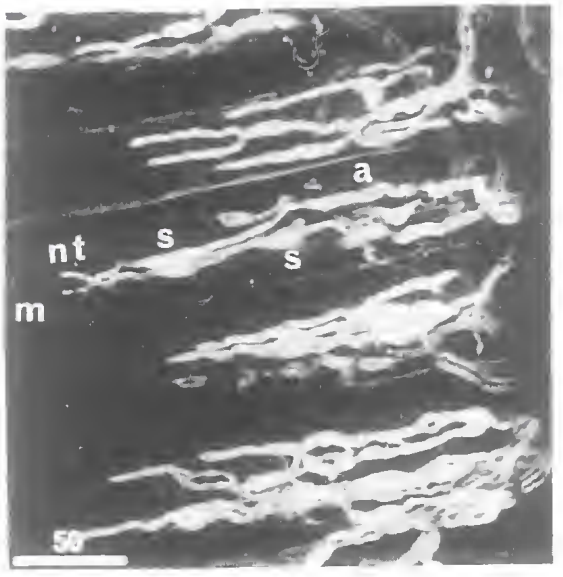

FIG.1. Low magnification view of motor nerve terminals (NT), Schwann cells (SC) and intramuscular axons (a) in a living frog cutaneous pectoris neuromuscular preparation. Notice the nonmyelinating Schwann cells covering the branches of the nerve terminals. Tridimensional reconstitution by a projection of 30 horizontal section series. The structures have been stained with the fluorescent membrane dye FM1-43 for 60min and then washed free of FM1-43.

Na^+-K^+-ATPase activity, it was suggested that the enhancement of neurotransmitter release may be due to a depolarization-induced Ca^{2+} influx (Bidard et al.,1984). CTX was reported also to enhance Ca^{2+}-dependent ACh release from pure cholinergic synaptosomes (Molgó et al.,1992b). If CTX depolarized synaptosomal membranes to levels above that needed to activate voltage-gated Ca^{2+} channels, then it would be expected that membrane depolarization, via Ca^{2+} influx, would contribute to this Ca^{2+}-dependent ACh release caused by CTX. However, blockade of Ca^{2+} channel subtypes in Torpedo synaptosomes (Moulian et al.,1993) by simultaneous application of FTX, a toxin purified from *Agelenopsis aperta* venom, synthetic omega-conotoxin and Gd^{3+} (Molgó et al.,1991b) did not prevent ACh release caused by CTX in the presence of Ca^{2+} (Molgó et al.,1993a). These results may suggest that CTX exerts its effects on ACh release from Torpedo synaptosomes by increasing synaptosomal Na^+ levels sufficiently to reverse the Na^+/Ca^{2+} exchange system which normally uses the Na^+ gradient to extrude Ca^{2+}. In the reversed mode the exchanger will import Ca^{2+}.

CTX also increases spontaneous quantal acetylcholine release at frog neuromuscular junctions even in a nominally Ca^{2+}-free medium supplemented with EGTA (Molgó et al.,1990). TTX completely prevented activation of the release process by CTX suggesting that the CTX effect depends on Na^+ entry into the terminal (Molgó et al.,1991a). Furthermore, ultrastructural studies performed at neuromuscular junctions in which quantal transmitter was exhausted irreversibly by CTX, after 3–4hr of toxin action, revealed a marked depletion of synaptic vesicles per nerve terminal cross-section. The depletion of synaptic vesicles was accompanied by enlargement of the presynaptic membrane coupled to the swelling of the terminal (Molgó et al.,1991a; Comella, Molgó & Legrand unpubl. results) suggesting that CTX impairs the recycling process that, under normal conditions, maintains the synaptic vesicle population during quantal release.

Experiments described here aim to characterize some of the basic changes occurring at the neuromuscular junction in situ during the action of CTX. For this purpose we have used a lipophilic dye, that becomes fluorescent only after incorporation into the outer leaflet of surface membranes, in conjunction with the recently evolved confocal laser scanning microscope which allows optical sectioning of the neuromuscular junction at a desired thickness and a subsequent 3-dimensional reconstitution of the imaged motor nerve terminals.

Confocal laser microscopy appears as one of the most exciting and valuable techniques for optical sectioning, high resolution three dimensional imaging and reconstitution of fluorescence-labelled or reflecting cellular structures. This kind of analysis can be done on living nerve-muscle preparations without the need of physical sectioning and enables the investigation of processes, like the time course of action of CTX, which is not readily studied in fixed preparations.

MATERIAL AND METHODS

Experiments were performed using isolated cutaneous pectoris nerve-muscle preparations from adult male frogs, *Rana esculenta* (20–25g) between October and April. The excised nerve-muscle preparation was pinned to the bottom of a rhodorsil-lined plexiglass chamber (2ml), exposed for 5–60min to the dye (FM1-43,

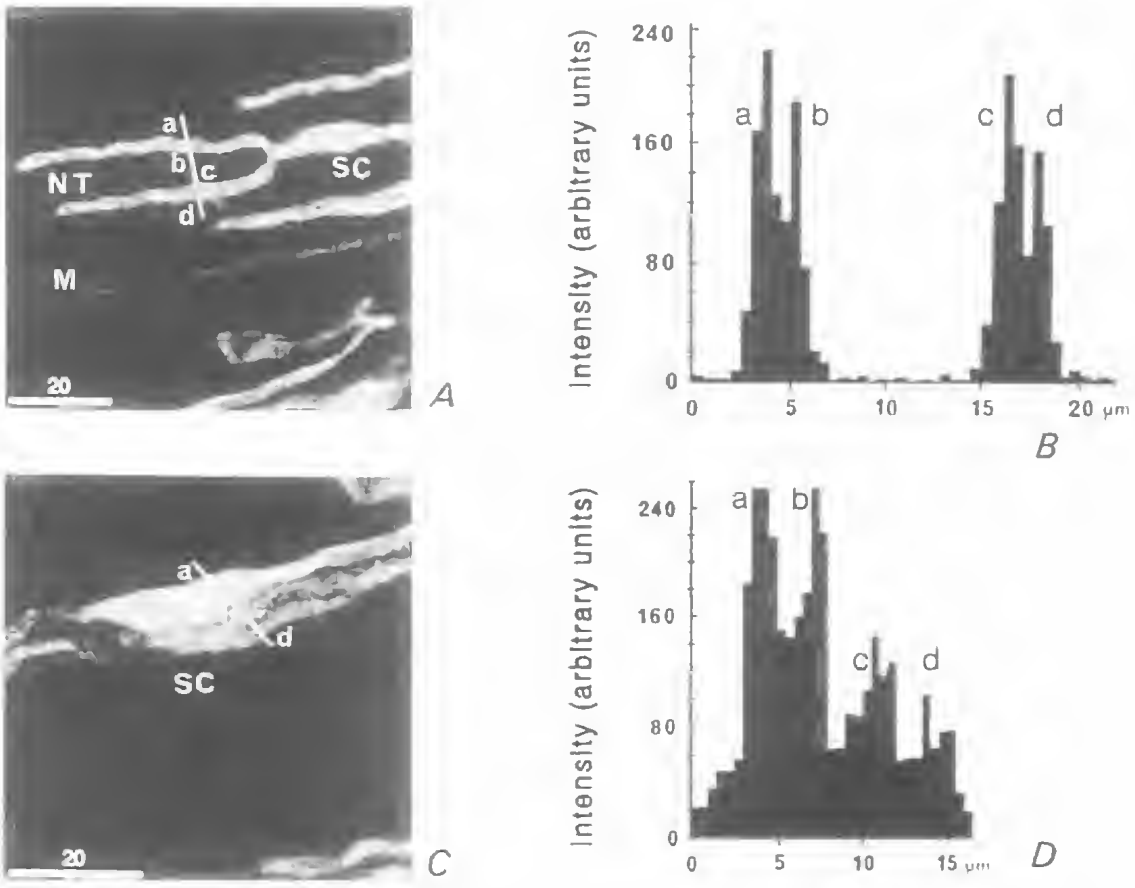

FIG.2. Images of a neuromuscular junction (A) and of a perisynaptic Schwann cell (C) stained with the dye FM1-43. In B and D, the intensity of the fluorescence between the lines shown in A and C is indicated. The peaks of the histograms in B and D (a,b,c,d) correspond to the zones labelled in the images A and C. The images A and C represent the 3-D reconstitution by a projection of 30 serial sections (0.5µm steps).

Molecular Probes, Eugene, Or., U.S.A) [N-(3-triethyl ammonium) propyl]-4-(dibutylamino-styryl pyridinium, dibromide (2µM) dissolved in standard physiological solution of the following composition (mM): NaCl, 115.0; KCl, 2.1; $CaCl_2$, 1.8; and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), 5 (pH=7.25); and then washed with the standard physiological solution. The experiments were carried out at 20°C. Only neuromuscular junctions of surface fibers were studied. In some experiments excitation contraction of cutaneous pectoris muscles was uncoupled by treating the preparations with 2M formamide (Sigma, St. Louis, U.S.A) as previously described (del Castillo & Escalona de Motta,1978). In other experiments, D-mannitol (Sigma, St. Louis, U.S.A) was added to the standard solution and osmolality was determined using a Knauer (Berlin, Germany) freezing-point osmometer. Ciguatoxin (CTX-1B) was extracted from *Gymnothorax javanicus* (moray eel) liver and viscera (Legrand et al.,1989; Murata et al., 1990). Tetrodotoxin was from Sigma (St. Louis, U.S.A.).

Neuromuscular junctions were imaged with a Sarastro-2000 confocal laser scanning microscope (Molecular Dynamics, California, U.S.A.) composed of an upwright NIKON optiphot-2 microscope equipped with a single argon-ion laser beam emmitting light at 488nm (high power, maximum output 25mW), with a 3% neutral density transmission filter to prevent dye bleaching. A 510nm dichroic mirror and a 510nm long pass emission filter were used. The aperture setting was 50µm. The photomultiplier was set at a constant level in a given experiment (between

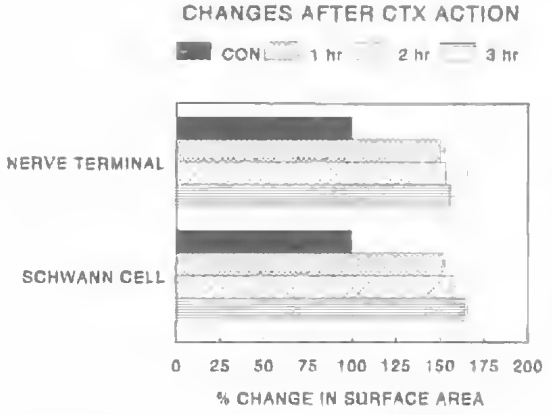

FIG.3. Relative changes caused by 10nM CTX-1b on the surface area of motor nerve terminals and Schwann cell somata with respect to controls at different times of toxin action. The black columns denote respective controls.

600–900V). Images were acquired with single scans or after averaging. Neuromuscular junctions were routinely visualized with a 40x water immersion objective (0.55 numerical aperture).

Control of the scanner module and image analysis of the data files was achieved with a Silicon Graphics workstation (Mountain View, Ca, U.S.A) integrated into the Sarastro system. Images were analyzed with a Silicon Graphics Personal Iris 4D/35G workstation using a UNIX™ operating system and the software Image Space from Molecular Dynamics. A series of optical sections were taken at 0.13–0.5µmsteps. Images from each experiment were processed identically and stored on rewritable magneto-optical disks. In all experiments neuromuscular junctions were imaged before and after the various treatments.

RESULTS AND DISCUSSION.

Staining of the Neuromuscular Junction in Situ

The fluorescent staining appears on motor nerve terminals, on myelinated nerve fibres, and in perisynaptic Schwann cells somata (Fig.1). This staining is difficult to wash out after such a long exposure (60min) to the dye. The mechanism of staining seems to be due to the high affinity of the dye for lipid membranes coupled with an inability to penetrate, so that the dye seems to partition only into the outer leaflet of surface membranes (Betz et al.,1992). In contrast to previous work by Betz et al. (1992), we have found that the FM1-43 dye also stains living motor nerve terminals in an activity-independent fashion. Staining in the various membrane structures was detected on resting preparations exposed for only 5min to the dye and then washed out, with dye-free medium. This staining lasted for more than 12hrs.

When the nerve terminal and the Schwann cell somata were imaged at higher magnification by a stack of horizontal scans, the image of the 3-D volume described by the section series (look through projection) revealed both surface and internal structures (Fig.2). The intensity of fluorescence was more marked at the contours and edges than in the interior of both structures. Pixel intensity plots of line scans (Fig.2b,d) showed peaks corresponding to the limits of the nerve terminal membrane and Schwann cell somata membrane. The axoplasma of the terminal and the cytoplasma of the Schwann cell exhibited lower intensity. Having characterized the dye staining in motor terminals and Schwann cells, we performed further experiments in order to determine whether CTX-1B was still active in enhancing quantal transmitter release after application and washout of the dye. Under these conditions, as in control junctions (see Molgó et al.,1990), CTX-1B (2.5nM) increased the frequency of miniature endplate potentials (data not shown). These results indicated first that the FM1-43 dye did not perturb the effect of CTX-1B and second that the dye was suitable for following eventual changes in the nerve terminal surface area during the action of CTX-1B

Effect of CTX-1b on Motor Nerve Terminals In Situ

In junctions in which muscle contraction was prevented by prior treatment with formamide, stained with the FM1-43 dye and then washed out, one of the nerve terminals was selected and, imaged before and after different times of CTX-1B (10nM) addition to the standard medium. Usually 10 horizontal section images (0.5µm step) were made for complete reconstructed view of the nerve terminals at each time period investigated. Increases in the nerve terminal surface area were evident within 15–17min of CTX-1B (10nM) addition to the medium, this increase in surface area continued for 3hrs. Relative changes in surface area at 1, 2 and 3hrs of CTX-1B action (Fig.3) were greatest during the first hour (50±2.0%; n=3) compared with the second and third hour of CTX-1B exposure. After the second and third hour nerve terminals only increased

3.4±0.15 and 5.7±0.29% respectively with respect to the first hour (Fig.3). When junctions were pretreated with TTX (1μM) no such changes were observed during 3hrs of CTX-1B action. Thus, the increase in nerve terminal surface area of motor nerve terminals might be related to both increase of intraterminal Na^+ concentration and to the enhanced quantal release. None of the 6 nerve terminals imaged during 3hrs with CTX-1B showed fluorescent spots inside the terminals, as observed with high K^+ medium (Betz et al., 1992). This result supports the previous view that CTX-1B blocks the recycling of clear synaptic vesicles (Molgó et al.,1991a).

Effect of CTX-1B on Perisynaptic Schwann Cells In Situ

Satellite cells of the nervous system, oligodendrocytes and astrocytes in the central nervous system and Schwann cells in the peripheral nervous system, have a regulatory role in synaptic transmission. Thus, glial cells can be depolarized by K^+ accumulation near active neurons in situ (Orkand et al.,1966), can respond to many chemical transmitters in vitro (Orkand,1982; Dave et al., 1991) and express a diversity of ion channels (Ritchie,1992; Sontheimer,1992). At the frog neuromuscular junction non-myelinating Schwann cells cover the motor nerve terminal and send fine processes around it (Birks et al., 1960; Dreyer & Peper,1974). The Schwann cell processes are generally located between the active zones at irregular intervals (Couteaux & Pécot Dechavassine,1970). We tested whether perisynaptic non-myelinating Schwann cells are affected, like motor nerve terminals, by CTX-1B (10nM). Schwann cells also markedly increased their surface area during 1, 2 and 3hrs of CTX-1B action (Fig.3). After 3 hours of CTX-1B action the increase was more marked (64±2.9%) than in motor nerve terminals. The changes of the Schwann cells lying directly over the nerve terminal were similar to the changes observed in cells located lateral to the nerve terminals. TTX (1μM) completely prevented such changes when applied before CTX-1B. Therefore, in addition to acting on motor nerve terminals CTX-1B also acts on Schwann cells. Is this related to the action of the CTX-1B on sodium channels of Schwann cells or is it the result of the changes in quantal acetylcholine release caused by CTX-1B? The role of the nonmyelinating perisynaptic Schwann cells at the frog neuromuscular junction during synaptic activity and particularly during transmitter release remains unknown. Recent studies have shown that motor nerve stimulation induces an increase in intracellular Ca^{2+} concentration in Schwann cells (Jahromi et al.,1992). Since Schwann cells undergo profound changes during the action of CTX-1B it is likely that they may play a role in the maintenance of the neuromuscular junction.

Effect of CTX-1B on Muscle Fibres In Situ

When skeletal muscle fibres in which muscle contraction was prevented (by formamide treatment) were imaged at junctional sites before and after 3hr of CTX-1Baction, the changes observed in muscle fibre surface area were of the order of 1−1.5% (n=4). Attempts to investigate the effects of CTX-1B in muscle fibres with functional excitation-contraction coupling failed due to the spontaneous contractions induced by the toxin which prevented imaging during the first hr of toxin action.

Effects of D-Mannitol after CTX-1B Action

Mannitol was reported to markedly improve neurologic and muscular dysfunction in patients with acute ciguatera (Palafox et al.,1988). Although these observations were uncontrolled, the dramatic clinical improvement suggested that mannitol may have a valuable therapeutic effect on this intoxication. The mechanism of action of mannitol is obscure (Russell & Egen,1991). One possibility that was suggested is that D-mannitol may neutralize the toxin by some covalent coupling or complexation. Another possibility is that mannitol may exert osmotic effects by increasing extracellular osmolality. Finally, one should take into account that mannitol reacts with free radicals and is considered as an effective hydroxyl radical scavenger (Halliwell & Gutteridge,1985). The possibility that hyperosmotic D-mannitol may exert its action on ciguatera due to its hydroxyl radical-scavenging properties or its water-draining effect has been suggested by Pearn et al., (1989).

A free radical is an atom or molecule that contains one or more unpaired electrons so that to attain stability either donates its electron to other molecules or acquires an extra electron from adjacent molecules. Indeed, free radicals are highly reactive and, because of their instability, damage cells and tissues. D-mannitol reacts with the very reactive and short-lived hydroxyl radical in a way that its concentration can be limited. Hydroxyl radicals also stimulate

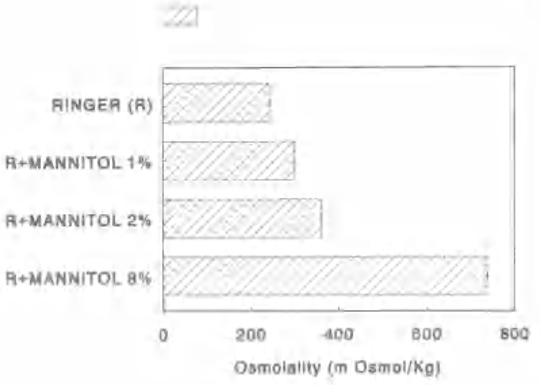

FIG.4. Osmolality of the standard Ringer's solution (R) and of the various standard solutions (R) to which D-mannitol was added.

phospholipase A_2 leading to release of arachidonic acid. One interesting property of hydroxyl radicals is their ability to initiate lipid peroxidation by extracting a hydrogen atom from polyunsaturated fatty acids such as arachidonic acid. This leads to cell membrane damage and frequently to cell death.

We performed experiments on isolated frog neuromuscular junctions in order to determine whether D-mannitol could modify the actions of CTX-1B. For this purpose, we used a dose of D-mannitol which has no osmotic effects per se but which protects kainate-induced death of cerebellar neurons in culture by scavenging hydroxyl radicals (Dykens et al.,1987). When preparations were pre-treated with 20μM D-mannitol, subsequent addition of CTX-1B (2.5nM) caused similar effects to those observed in the absence of D-mannitol i.e. there was an increase of spontaneous quantal release and spontaneous asynchronous contractions (data not shown) and depolarization of the muscle membrane (Molgó et al.,1990). Thus, it appears that 20μM D-mannitol does not prevent the actions of CTX-1B at the neuromuscular junction. When nerve terminals and Schwann cells were imaged during 3hrs of CTX-1B action with the confocal laser scanning microscope the typical changes above reported, i.e. changes in surface area of nerve terminals and Schwann cells, were observed.

We conclude that D-mannitol concentrations which has been reported to exert an effective hydroxyl radical-scavenger action neither prevented binding of CTX-1B nor antagonized effects of the toxin.

Further experiments were performed with higher concentrations of D-mannitol (54.9mM =1%; 109.8mM=2% and 439.1mM =8%) added to the standard Ringer's solution to determine osmotic effects of this agent. Since solutions containing 1, 2 and 8% D-mannitol added to the standard Ringer solution (Fig.4) had osmolalities that are 24% (1% D-mannitol), 48.8% (2% D-mannitol) and 203.7% (8% D-mannitol) higher than the standard Ringer's solution and, increases in osmotic pressure causes dramatic increases in spontaneous quantal release. At the frog neuromuscular junction a 50% increase in osmotic pressure by addition of sucrose causes a reversible 45-fold increase in miniature endplate potential frequency, as previously reported (Fatt & Katz,1952). We did not attempt to study the effects of CTX-1B in the presence of such high concentrations of mannitol. Instead we tried D-mannitol after CTX-1B action at the neuromuscular junction to determine whether this agent at different osmolaties could modify the changes in the nerve terminal and Schwann cells previously described with the toxin. D-mannitol effectively caused a shrinkage of nerve terminals and Schwann cells previously swollen by the action of CTX-1B (Fig.5). When the effects of mannitol were quantified in nerve terminals it was evident that 2% mannitol applied for 30min decreased the nerve terminal surface area by 21±1.0% and the Schwann cell surface area by 15.2%, while the muscle fibre was decreased by 11.4±O.2% (n=4). In control junctions D-mannitol reduced the nerve terminal surface area by only 13±1.2%, the Schwann cell surface area by 10±0.5% and the muscle surface area by 11±0.6% indicating that mannitol was more effective in reducing nerve terminal surface area in nerve terminals treated with CTX-1B than in control nerve terminals. However, muscle changes caused by mannitol were no different in CTX-1B treated junctions as compared to controls. D-mannitol, at concentrations that increased the osmolality of the standard solution by c.50%, reversed swelling of motor nerve terminals and Schwann cells observed during long-term effects of CTX-1B.

These findings are important since the clinical improvement observed in acute ciguatera after mannitol treatment may be ascribed to the osmotic action exerted by this agent in the peripheral nervous system and skeletal muscle fibres, which would result in a shift of water from the intracellular to the extracellular compartment.

DISCUSSION

Cell swelling of motor nerve terminals and perisynaptic Schwann cells was a common

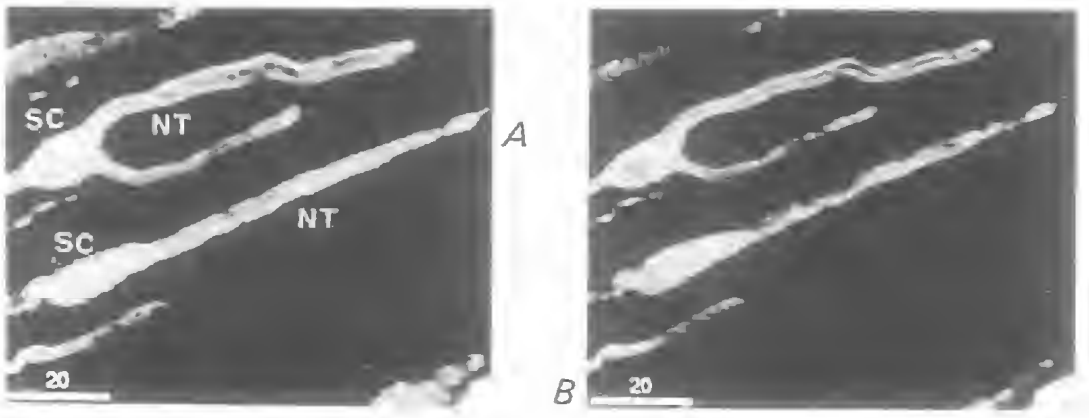

FIG.5. Nerve terminal (NT) and perisynaptic Schwann cell somata (SC) from a junction treated for 3hr with 2.5nM CTX- 1B (a) and after 30min of D-mannitol (2%) added to the standard solution (b). Note the shrinkage of structures after mannitol action.

response to CTX-1B application at living neuromuscular junctions. Imaging methods are the only way in which the shape changes accompanying cell volume changes can be determined. However, determinations of cell volume are not easy, even in a relatively simple synapse as the neuromuscular junction. The term cell volume is a complex concept because neither motor endings nor perisynaptic Schwann cells have simple individual geometric shapes and relationships. Furthermore, the mechanisms of volume homeostasis in motor endings have not been explored.

Changes in nerve terminal volume caused by CTX-1B may result from the fusion of synaptic vesicles to the presynaptic membrane and the influx of Na^+ across the presynaptic membrane. Previous electron microscopic studies of motor endings in fixed specimens revealed time-dependent increase in the nerve terminal perimeter, alterations in nerve terminal mitochondria and profound depletion of synaptic vesicles after CTX-1B action (Molgó et al., 1991; Molgó, Comella & Legrand, unpubl.).

The Na^+ content of the nerve terminals is expected to be increased by CTX-1B. Under normal conditions, water is in thermodynamic equilibrium across the terminal membrane. However, any change in the intracellular Na^+ concentration will result in a rapid water flow from the extracellular to the intracellular compartment. Because the nerve terminal is readily distensible, transmembrane water movements will result in nerve terminal swelling. Schwann cell somata swelling in situ is probably also related to the increase in Na^+ concentration through activation of sodium channels sensitive to the action of both CTX-1B and TTX. The contribution of enhanced quantal transmitter release to the swelling of Schwann cells remains to be established.

D-Mannitol at concentrations reported to exert an effective hydroxyl radical scavenger action neither prevented the action of CTX-1B nor antagonized its effects. However, at higher concentrations mannitol exerted osmotic effects that caused shrinkage of both motor nerve terminals and Schwann cell somata previously swollen by the action of CTX-1B probably by shifting water from the intracellular to the extracellular compartment.

This report demonstrates that CTX-1B causes time dependent changes in the surface area of motor nerve terminals and perisynaptic Schwann cells in living neuromuscular junctions. We have shown that confocal laser microscopy is a new tool for research on the effects of ciguatoxins on living tissues. While the extent of its future applications in the field of the ciguatoxins is hard to predict, its potential for neurobiological research appears enormous.

ACKNOWLEDGEMENTS

We are grateful to Dr Spencer Brown, Institut des Sciences Végétales C.N.R.S. at Gif sur Yvette for cogent advice concerning the use of the confocal microscope and for stimulating discussions concerning the analysis of data. P.J. is supported by a fellowship from Direction des Recherches Etudes et Techniques (D.R.E.T.). This work has been supported by grant 91/090 from D.R.E.T.

LITERATURE CITED

ANDERSON, D.M. & LOBEL, P.S. 1987. The continuing enigma of ciguatera. Biological Bulletin 172: 89–107.

BADEN, D.G. 1989. Brevetoxins: unique polyether dinoflagellate toxins. FASEB Journal 3: 1807–1817.

BAGNIS, R., KUBERSKI, T. & LAUGIER, S. 1979. Clinical observations on 3,009 cases of ciguatera (fish poisoning) in the South Pacific. American Journal of Tropical Medicine and Hygiene 28: 1067–1073.

BENOIT, E., LEGRAND, A.M. & DUBOIS, J.M. 1986. Effects of ciguatoxin on current and voltage clamped frog myelinated nerve fibre. Toxicon 24: 357–364.

BETZ, W.J., MAO, F. & BEWICK, G.S. 1992. Activity- dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. Journal of Neuroscience 12: 363–375.

BIDARD, J.N., VIJVERBERG, H.P.M., FRELIN, C., CHUNGUE, E., LEGRAND, A.M., BAGNIS, R. & LAZDUNSKI, M. 1984. Ciguatoxin is a novel type of Na^+ channel toxin. Journal of Biological Chemistry 259: 8353–8357.

BIRKS, R., HUXLEY, H.E. & KATZ, B. 1960. The fine structure of the neuromuscular junction of the frog. Journal of Physiology (London) 150: 134–144.

COUTEAUX, R. & PECOT-DECHAVASSINE, M. 1970. Vésicules synaptiques et poches au niveau des 'zones actives' de la jonction neuromusculaire. Comptes Rendus de l'Académic des Sciences, Paris, Série D. 271: 2346–2349.

DAVE, V., GORDON, G.W. & MCCARTHY, K.D. 1991. Cerebral type 2 astroglia are heterogeneous with respect to their ability to respond to neuroligands linked to calcium mobilization. Glia 4:440–447.

DEL CASTILLO, J. & ESCALONA DE MOTTA, G. 1978. A new method for excitation-contraction uncoupling in frog skeletal muscle. Journal of Cell Biology 78:782–784.

DREYER, F. & PEPER, K. 1974. A monolayer preparation of innervated skeletal muscle fibres of the m. cutaneous pectoris of the frog. Pflügers Archiv 348:257–262.

DYKENS, J.A., STERN, A. & TRENKNER, E. 1987. Mechanism of kainate toxicity to cerebellar neurons in vitro is analogous to reperfusion tissue injury. Journal of Neurochemistry 49: 1222–1228.

FATT, P. & KATZ, B. 1952. Spontaneous subthreshold activity at motor nerve endings. Journal of Physiology (London) 117: 109–128.

GAWLEY, R.E., REIN, K.S., KINOSHITA. M. & BADEN, D.G. 1992. Binding of brevetoxins and ciguatoxin to the voltage-sensitive sodium channel and conformational analysis of brevetoxin B. Toxicon 30: 780–785.

GILLESPIE, N.C., LEWIS, R.J., PEARN, J. et al. 1986. Ciguatera in Australia: occurrence, clinical features, pathophysiology and management, Medical Journal of Australia 145: 584–590.

HALLIWELL, B. & GUTTERIDGE, J.M.C. 1985. The role of transition metals in superoxide-mediated toxicology.Pp.45–82. In L.W. Oberley, ed., 'Superoxide Dismutase, Vol. III'. (CRC Press: Boca Raton, Florida).

JAHROMI, B.S., ROBITAILLE, R. & CHARLTON, M.P. 1992. Transmitter release increases intracellular calcium in perisynaptic Schwann cells in situ. Neuron 8: 1069–1077.

LEGRAND, A.M., LITAUDON, M., GENTHON, J.N., BAGNIS, R. & YASUMOTO, T. 1989. Isolation and some properties of ciguatoxin. Journal of Applied Phycology 1: 183–188.

LEWIS, R.J. & SELLIN, M. 1992. Multiple ciguatoxins in the flesh of fish. Toxicon 30: 915–919.

LEWIS, R.J,. SELLIN, M., POLIN M.A., NORTON, R.S., MACLEOD, J.K. & SHEIL, M.M. 1991. Purification and characterization of ciguatoxins from moray eel (*Lycodontis javanicus*, Muraenidae). Toxicon 29: 1115–1127.

LOMBET, A., BIDARD, J.N. & LAZDUNSKI, M. 1987. Ciguatoxin and brevetoxins share a common receptor site on the neuronal voltage-dependent Na^+ channel. FEBS Letters 219: 355–359.

MOLGÓ, J., COMELLA, J.X. & LEGRAND, A.M. 1990. Ciguatoxin enhances quantal transmitter release from frog motor nerve terminals. British Journal of Pharmacology 99:695–700.

MOLGÓ, J., COMELLA, J.X., SHIMAHARA, T. & LEGRAND, A.M. 1991a. Tetrodotoxin-sensitive ciguatoxin effects on quantal release, synaptic vesicle depletion, and calcium mobilization. Annals of the New York Academy of Sciences 635: 485–489.

MOLGÓ, J., DEL POZO, E., BAÑOS, J.E. & ANGAUT-PETIT, D. 1991b. Changes of quantal transmitter release caused by gadolinium ions at the frog neuromuscular junction. British Journal of Pharmacology 104: 133–138.

MOLGÓ, J., BENOIT, E., COMELLA, J.X. & LEGRAND, A.M. 1992a. Ciguatoxin: a tool for research on sodium-dependent mechanisms. Pp.149–164. In P.M. Conn, (ed.), 'Methods in neuroscience, vol.8, Neurotoxins'. (Academic Press: New york).

MOLGÓ, J., SHIMAHARA, T., MOROT GAUDRY-TALARMAIN Y., COMELLA, J.X. & LEGRAND, A.M. 1992b. Ciguatoxin-induced changes in acetylcholine release and in cytosolic calcium levels. Bulletin de la Société de Pathologie Exotique 85: 486–488.

MOLGÓ, J., MOROT GAUDRY-TALARMAIN, Y., LEGRAND, A.M. & MOULIAN, N. 1993a. Ciguatoxin extracted from poisonous moray eels (Gymnothorax javanicus) triggers acetylcholine release from Torpedo cholinergic synaptosomes via reversed Na^+-$Ca2^+$ exchange. Neuroscience Letters 160: 65–68.

MOLGÓ, J., SHIMAHARA, T. & LEGRAND, A.M. 1993b. Ciguatoxin, extracted from poisonous morays eels, causes sodium-dependent calcium mobilization in NG108-15 neuroblastoma x glioma hybrid cells. Neuroscience Letters 158: 147–150.

MOULIAN, N. & MOROT-GAUDRY-TALARMAIN, P. 1993. *Agelenopsis aperta* venom and FTX, a toxin purified from it, inhibit acetylcholine release in Torpedo synaptosomes. Neuroscience 54: 1035–1041.

MURATA, M., LEGRAND A.M., ISHIBASHI, Y. & YASUMOTO, T. 1989. Structures and configurations of ciguatoxin and its congener. Journal of the American Chemical Society 111: 8929–8931.

MURATA, M., LEGRAND A.M., ISHIBASHI, Y., FUKUI, M. & YASUMOTO, T. 1990. Structures and configurations of ciguatoxin from the Moray eel *Gymnothorax javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus toxicus*. Journal of the American Chemical Society 112: 4380–4386.

ORKAND, R.K. , NICHOLLS, J.G. & KUFFLER, S.W. 1966. Effect of nerve impulses on the membrane potential of glial cells in the central nervous system of amphibia. Journal of Neurobiology 29: 788–806.

ORKAND, R.K. 1982. Signalling between neuronal and glial cells. Pp.147–157. In T. A. Sears, (ed.). 'Neuronal-glial cell interrrelationships'. (Springer-Verlag: New York).

PALAFOX, N.A., JAIN, L.G., PINANO, A.Z., GULICK, T.M., WILLIAMS, R.K. & SCHATZ, I.J. 1988. Successful treatment of ciguatera fish poisoning with intravenous mannitol. Journal of the American Medical Association 259: 2740–2742.

PEARN, J.H., LEWIS, R.J. & RUFF, T. et al. 1989. Ciguatera and Mannitol: experience with a new treatment regimen. Medical Journal of Australia 151:77–80.

RITCHIE, J.M. 1992. Voltage-gated ion channels in Schwann cells and glia. Trends in Neurosciences 15: 345–350.

RUSSELL, F.E. & EGEN, N.B. 1991. Ciguateric fishes, ciguatoxin (CTX) and Ciguatera poisoning. Journal of Toxicolology-Toxin Reviews 10, 37–62.

SCHEUER, P.J., TAKAHASHI, W., TSUTSUMI, J. & YOSHIDA, T. 1967. Ciguatoxin isolation and chemical nature. Science 155: 1267–1268.

SONTHEIMER, H. 1992. Astrocytes, as well as neurons, express a diversity of ion channels. Canadian Journal of Physiology and Pharmacology 70: S223–S238.

TACHIBANA, K., NUKINA, M., JOH, Y. & SCHEUER, P. 1987. Recent developments in the molecular structure of ciguatoxin. Biological Bulletin 172: 122–127.

SWIFT A.E.B. & SWIFT, T.R. 1993. Ciguatera. Journal of Toxicology-Clinical Toxicology 31: 1–29.

WITHERS, N.W. 1982. Ciguatera fish poisoning. Annual Review of Medicine 33: 97–111.